REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	12 NOV 2000	Final report: 15 AUG 1997 -- 14 AUG 2000

4. TITLE AND SUBTITLE
Cloning and Expression of Human Chromium-Reducing Enzymes

5. FUNDING NUMBERS
G
F49620-97-1-0423

6. AUTHOR(S)
Charles R. Myers, Ph.D., Associate Professor

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Medical College of Wisconsin
Dept. of Pharmacology & Toxicology
8701 Watertown Plank Rd
Milwaukee, WI 53226

8. PERFORMING ORGANIZATION REPORT NUMBER
2200732-final-1

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Air Force Office of Scientific Research/NL
110 Duncan Avenue, Suite B115
Bolling AFB, DC 20332-0001

AASERT: Augmentation Award for Scientific and Engineering Research Training

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

DISTRIBUTION STATEMENT A
Approved for Public Release
Distribution Unlimited

12a. DISTRIBUTION / AVAILABILITY STATEMENT
The author and performing organization do not have any objections if the AFOSR wishes to make the contents of this report available to the public.

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

The cloning and expression in *E. coli* of three of the proteins of interest (FMO3, P450 reductase, and b_5 reductase) were accomplished. Human cytochrome b_5 and P450 reductase became available from commercial sources. Recombinant FMO3 had little to no NADPH-dependent Cr(VI) reduction activity; similarly, when tested alone, none of the other proteins (cytochrome b_5, P450 reductase, b_5 reductase) had prominent Cr(VI) reductase activity. Efficient electron transfer from NADPH to cytochrome b_5 was observed using proteoliposomes containing human recombinant cytochrome b_5 and P450 reductase. When normalized to equivalent cytochrome b_5 concentrations, the NADPH-dependent Cr(VI) reduction rates mediated by these proteoliposomes were essentially identical to those for human microsomes. Trace amounts of iron (Fe) could dramatically stimulate Cr(VI) reduction by these proteoliposomes; this stimulation could be abolished by deferoxamine. The Fe(III) reduction rates were sufficient to account for the Fe-mediated stimulation of Cr(VI) reduction. Cr(V) was detected as a transient intermediate formed during NADPH-dependent Cr(VI) reduction mediated by these proteoliposomes. Iron also stimulated the subsequent reduction of Cr(V) by these proteoliposomes which would accelerate the formation of Cr(IV), a highly reactive species. Under aerobic conditions, Cr(VI) reduction by these proteoliposomes resulted in the generation of hydroxyl radical (•OH), a highly damaging species.

Overall, the interaction of cytochrome b_5 with P450 reductase can account for: (i) essentially all of the NADPH-dependent Cr(VI) reduction seen with human microsomes; (ii) the iron-mediated stimulation of Cr(VI) reduction; and (iii) the generation of reactive species [e.g. Cr(V), •OH] which are likely involved in some of the cytotoxic and genotoxic effects associated with Cr(VI) exposure.

14. SUBJECT TERMS
chromium, toxicity, iron, human, reductive activation, P450 reductase, cytochrome b5,

15. NUMBER OF PAGES
29

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18

TABLE OF CONTENTS

Final Technical Report (Cumulative for 15 AUG 1997 – 14 AUG 2000)
AASERT/AFOSR Grant no. F49620-97-1-0423
CLONING AND EXPRESSION OF HUMAN CHROMIUM-REDUCING ENZYMES
Principal Investigator: Charles R. Myers, Ph.D., Medical College of Wisconsin
Program Manager: Dr. Walter J. Kozumbo, AFOSR/NL

SIGNIFICANCE:

Exposure to chromium (Cr) compounds is associated with a wide array of toxic effects, including serious damage to internal organs, increased incidence of certain cancers, pulmonary fibrosis and chronic bronchitis, skin ulcers and allergic dermatitis, and impairment of primary immune responses. The inhalation of Cr-containing fumes, dusts, and particles is primarily associated with respiratory tract damage, whereas exposure to forms which are internalized is associated with toxicity to other organs including liver and kidneys. The relative risk of lung cancer in ex-chromate workers whose exposure duration was >9 years is 21.6, whereas that in the general male smoking population is 3–4.7, implying that Cr is a much more potent lung carcinogen than cigarette smoke (1).

Occupational uses in which significant Cr exposure can occur include the production and use of chromate pigments, the machining and welding of stainless steel and other high tensile structural steels used in jet engines, zinc chromate primer paints used on aircraft, chrome plating, offset printing, photography, the production and use of certain cleaning agents, and others.

The intracellular reduction Cr(VI) to Cr(III), and the resulting generation of reactive species, is thought to play a key role in the cytotoxicity, mutagenicity, and carcinogenicity of Cr compounds. An understanding of Cr(VI) reduction mechanisms is therefore central to understanding Cr-mediated toxicity. Our findings have shown that rodent models on this subject cannot be extrapolated to humans as there are key differences between Cr(VI) reduction in these systems (2, 3). Based on preliminary studies, four microsomal enzymes emerged as possible candidates for mediators of Cr(VI) reduction in human tissue: NADPH:P450 reductase, flavin-containing monooxygenase (FMO), cytochrome b_5 and NADH:b_5 reductase.

The primary goal of our work is to identify the components responsible for Cr(VI) reduction in human tissue. The tools needed to accomplish these goals are the purified proteins of interest. The major human hepatic FMO (FMO3) has not yet been purified from human tissue, and the other human enzymes of interest were not commercially available. The cDNA sequences of these proteins were known, however. An expression system from which active enzyme can be purified provides an alternative and attractive approach for obtaining the necessary enzymes. It also provides a way to assess their activities separate from the complex mixture of proteins present in human microsomes. Cloning vectors which facilitated affinity purification of expressed enzymes while preserving their activity were available.

ORIGINAL OBJECTIVES:

The original specific aim of this project was to provide a graduate student with training in molecular toxicology as it applied to an AFOSR project on the mechanisms of Cr(VI) reduction in human hepatic tissue. The student will work under the direct supervision of the P.I. to learn and apply the necessary techniques to clone and express human FMO3, P450 reductase, cytochrome b_5 and cytochrome b_5 reductase. The expressed proteins will be purified using a targeted affinity chromatography resin, and used as antigens to generate antibodies and as purified enzymes to assess their potential roles in Cr(VI) and iron reduction.

OVERVIEW OF OBJECTIVES ACCOMPLISHED:

The cloning and expression in *E. coli* of three of the proteins of interest (FMO3, P450 reductase, and b_5 reductase) has been accomplished. Sufficient amounts of all three proteins were obtained for use as antigens and for preliminary activity studies. The expression of cytochrome b_5 was underway when it became available commercially. At about the same time, human P450 reductase also became available from commercial sources. The costs of purchasing P450 reductase and cytochrome b_5 were actually less than what it cost us to generate them from recombinant sources, so commercial enzymes were used for subsequent experiments with P450 reductase and cytochrome b_5. Experiments using b_5 reductase and FMO3 were done using purified proteins we generated from recombinant sources.

Using these purified proteins, we demonstrated that there was marked stimulation of NADH-dependent Cr(VI) reduction by the addition of purified cytochrome b_5 and b_5 reductase to human microsomes. Similarly, the addition of purified cytochrome b_5 to human microsomes significantly stimulated Cr(VI) reduction (4).

While recombinant FMO3 displayed prominent methyl *p*-tolyl sulfide oxidation activity expected for FMO3, it had little to no NADPH-dependent Cr(VI) reduction activity under aerobic or anaerobic conditions. Similarly, when tested alone, none of the other three proteins (cytochrome b_5, P450 reductase, b_5 reductase) had prominent Cr(VI) reductase activity. When human liver microsomes were subjected to isopycnic sucrose gradient ultracentrifugation, fractions which contained both cytochrome P450 reductase and cytochrome b_5, or both cytochrome b_5 and b_5 reductase, accounted for the vast majority of NADPH- or NADH-dependent Cr(VI) reduction activity, respectively.

Since cytochrome b_5 cannot directly receive reducing equivalents from NADPH or NADH, P450 reductase or b_5 reductase, respectively, are required to transfer reducing equivalents to cytochrome b_5. Efficient electron transfer from NADPH to cytochrome b_5 was confirmed by spectral analysis using proteoliposomes containing human recombinant cytochrome b_5 and P450 reductase. When normalized to equivalent cytochrome b_5 concentrations, the NADPH-dependent Cr(VI) reduction rates mediated by these proteoliposomes were essentially identical to those for human microsomes (5). Similar to microsomes, trace amounts of iron (Fe) could dramatically stimulate Cr(VI) reduction by these proteoliposomes; this stimulation could be abolished by deferoxamine, a specific Fe(III) chelator (5). The NADPH-dependent reduction rates of various Fe(III) complexes were sufficient to account for the Fe-mediated stimulation of Cr(VI) reduction. Cr(V) was detected by electron spin resonance (ESR) spectroscopy as a transient intermediate formed during NADPH-dependent Cr(VI) reduction mediated by these proteoliposomes. Iron also stimulated the subsequent reduction of Cr(V) by these proteoliposomes which would accelerate the formation of Cr(IV), a highly reactive species. Under aerobic conditions, Cr(VI) reduction by these proteoliposomes resulted in the generation of hydroxyl radical (•OH), a highly damaging species; this is likely the result of Cr(V) and/or Cr(IV) mediated Fenton-type chemistry.

Overall, none of the four microsomal proteins examined were able to mediate significant Cr(VI) reduction on their own. However, the interaction of cytochrome b_5 with P450 reductase can account for essentially all of the NADPH-dependent Cr(VI) reduction seen with human microsomes. The interaction of these two proteins also accounts for the iron-mediated stimulation of Cr(VI) reduction, and the generation of reactive species [e.g. Cr(V), •OH] which are likely involved in some of the cytotoxic and genotoxic effects associated with Cr(VI) exposure. Since cytochrome b_5 is the reductant of Cr(VI) and Fe(III), we predict that the interaction of b_5 reductase with cytochrome b_5 will yield similar results for NADH-dependent Cr(VI) reduction.

The generation and use of these recombinant proteins has greatly extended our understanding of the mechanisms and consequences of microsomal Cr(VI) reduction, and further supports the prediction that simultaneous exposure to Cr(VI) and iron (or to compounds which increase available intracellular iron) represents an additional risk factor with potentially serious toxicologic consequences.

ACCOMPLISHMENTS, NEW FINDINGS, AND THEIR IMPLICATIONS:

Goal #1 — Cloning and Expression of Human Microsomal Enzymes:

Generation of Recombinant Human Cytochrome b_5 Reductase. The cDNA for cytochrome b_5 reductase was amplified from a human liver cDNA library (Clontech) using specific primers and the Expand High Fidelity PCR system (Boehringer-Mannheim, Indianapolis, IN). *Nco*I and *Bam*HI sites were added to the 5′ ends of the forward and reverse primers, respectively. The resulting PCR product was ligated into pCR2.1 (Invitrogen, Carlsbad, CA). DNA sequence analysis confirmed that the PCR product coded for b_5 reductase. The cDNA for b_5 reductase was excised from pCR2.1 by double digestion with *Nco*I and *Bam*HI, and ligated into the expression vector pCAL-c (Stratagene, La Jolla, CA) which contains an in-frame calmodulin-binding domain (CBD) at its C-terminus. This construct was transformed into *E. coli* BL21(DE3)pLysS for subsequent expression in LB broth. Several parameters were varied to optimize expression from this clone, and, after the optical density of the culture had reached 0.6–1.0 at 600 nm, 12 hr induction with 1 mM IPTG at 30°C under aerobic conditions was found to yield the greatest expression. The cells were harvested by centrifugation. While Triton X-100, Sarkosyl and Nonidet P-40 were largely ineffective, the detergent B-PER (Pierce) (4°C for 1 hr to lyse the cells and solubilize the proteins) resulted in good solubilization of the fusion from the cell pellet. The solubilized extract was then applied to a 5′ADP agarose column (N^6-linked) which had been pre-equilibrated with 20 mM Tris-acetate buffer, pH 7.5, containing 1 mM EDTA. The column was processed to remove non-adherent proteins, and b_5 reductase was eluted with 50 mM Tris-acetate buffer, pH 8.1, containing 1 mM EDTA, 0.05% Triton X-100, 1.5 M NaCl, 0.5 mM PMSF and 2 mM ADP. Subsequent purification steps were attempted to eliminate exisiting contaminants, and included calmodulin affinity chromatography, 5′-AMP affinity chromatography, ion exchange chromatography using DEAE or hydroxyapatite, and PEG (polyethylene glycol) fractionation. In some cases (e.g. calmodulin affinity) the strategy was unsuccessful. Other combinations of steps did eliminate several contaminants.

Activity studies were conducted with the mostly pure b_5 reductase-CBD fusion. The fusion displayed prominent activity for NADH-dependent reduction of ferricyanide (6), a standard assay for b_5 reductase; the assay was linear, and the purified b_5 reductase fusion displayed an activity of 0.7×10^6 to 2.5×10^6 nmol/min/mg. As expected, the activity of the b_5 reductase fusion could be completely inhibited by *p*-hydroxymercuribenzoate. However, the b_5 reductase fusion displayed only a very slow rate of Cr(VI) reduction, and was unable to reduce any of several iron(III) compounds, including Fe(III)-citrate, ammonium-Fe(III)-citrate, Fe(III)-DETPA, Fe(III)-phosphate, Fe(III)-acetylacetonate, Fe(III)-ADP, Fe(III)-ATP, Fe(III)-EDTA, and Fe(III)-EDDA. This suggests that it cannot act alone to mediate Cr(VI) reduction either directly, or indirectly via iron reduction. However, the addition of 3 μg b_5 reductase-CBD to human hepatic microsomes (from human N, 0.27 mg protein) increased NADH-dependent Cr(VI) reduction by approximately 20%. The simultaneous addition of 4.65 μg cytochrome b_5 + 3 μg b_5 reductase-CBD to these same microsomes increased NADH-dependent Cr(VI) reduction by 52%. These results suggest that b_5 reductase likely acts together with cytochrome b_5 to mediate Cr(VI) reduction; these proteins did not interact in solution, however, consistent with previous reports that they require a lipid environment to facilitate proper protein alignment for electron flow from b_5 reductase to cytochrome b_5.

To facilitate more complete purification, while still retaining activity, we explored other potential expression system for b_5 reductase. One was the vector pET-21d, which contains an in-frame hexahistidine sequence at its C-terminus. While this fusion was generated and the enzyme was active, the nickel affinity column did not bind the fusion very efficiently so purification was not readily feasible. This was the case for three parent strains of *E. coli* that were used [AD494(DE3)pLysS, BL21(DE3)pLysS, TB1].

Another approach involved the use of pMALc (New England BioLabs), which contains an in-frame 50-kDa maltose-binding protein (MBP) at its N-terminus. After successfully optimizing

conditions for expression of this fusion clone, sufficient MBP-b_5 reductase extract was generated. The fusion exhibited limited binding to the maltose column so only partial purification was possible using this strategy. Activity of b_5 reductase increased after treatment of the fusion with Factor Xa, which removes the N-terminal maltose-binding domain, but the bacterial proteases themselves also mediated some of this cleavage despite the presence of protease inhibitors. While additional columns (above) increased the purity of the fusion, it lost activity through the various steps, so partially pure preparations had to be used. The MBP-b_5 reductase fusion could not reduce Cr(VI) or iron, nor could it interact in solution with cytochrome b_5 to mediate iron reduction. Such interactions will have to be tested in artificial liposome environments (see below).

Sufficient b_5 reductase-CBD fusion was purified for use as an antigen. The polyclonal antibody generated against this fusion was specific for b_5 reductase in Western blots. However, it did not prove sufficient for immunopurification of b_5 reductase either from human microsomes or from the *E. coli* expression strains. This antibody did exhibit a concentration-dependent inhibition of ferricyanide reductase activity of the b_5 reductase-CBP fusion, but it does not block electron transfer to cytochrome b_5 in microsomes or the NADH-dependent Cr(VI) reduction by microsomes.

Generation of Recombinant Human FMO3. Since the major human FMO isoform, FMO3, has not been successfully purified from human liver, an approach was developed to clone and express FMO3. Using specific primers and a human liver cDNA library template, PCR was used to generate the cDNA for FMO3; this cDNA was cloned into the expression vector pCALc (Stratagene) which contains an in-frame calmodulin-binding domain (CBD) at its C-terminus. After successfully optimizing conditions for expression from this clone, sufficient FMO3-CBD fusion was purified for use as an antigen and for activity studies. While the polyclonal antibody generated against the FMO3-CBD fusion was specific for FMO in Western blots, it did not inhibit the FMO activity of human microsomes. The purified FMO3-CBD fusion did not display significant FMO activity (oxidation of methyl *p*-tolyl sulfide), so it could not be used directly to assess Cr(VI)- or Fe(III)-reducing activity.

An alternate bacterial expression approach was therefore developed. Human FMO3 cDNA was cloned into pMALc (New England BioLabs), which contains an in-frame maltose-binding protein (MBP) at its N-terminus. After successfully optimizing conditions for expression of this fusion clone, sufficient MBP-FMO3 was purified for use as an antigen and for activity studies. MBP-FMO3 had prominent FMO activity (methyl *p*-tolyl sulfide oxidation), but did not reduce Cr(VI) or any of the Fe(III) complexes that were examined. This suggests that FMO3 is not a significant player in human microsomal Cr(VI) reduction.

Generation of Recombinant Human P450 Reductase. A bacterial expression system approach based on pCALc (above) was utilized for human P450 reductase. After optimizing conditions for its expression, sufficient material was purified, using affinity chromatography, for use as an antigen and in activity studies. When normalized per unit of cytochrome *c* reductase activity (the standard assay for P450 reductase activity), purified P450 reductase was a relatively poor reducer of Fe(III) or Cr(VI) when compared to human microsomes. This supports our previous results which suggested that P450 reductase cannot act alone to mediate high rates of Fe(III) and Cr(VI) reduction, but that it likely acts in concert with other microsomal components, e.g. cytochrome b_5. Polyclonal antisera raised against purified P450 reductase inhibited the microsomal reduction of cytochrome *c* by approximately 50%, and the reduction of iron(III) by 23–32%.

Methods Used to Accomplish Goal #1:

Molecular Biology Procedures. All procedures for PCR, cloning, subcloning, DNA sequencing, etc. were done using previously described standard procedures (7–9), following manufacturers' recommendations as appropriate.

Cr(VI) reduction assay. Experiments to assess Cr(VI) reductase activity were conducted as previously described (2), using an NADPH-regenerating system (3 mM $MgCl_2$, 1 mM NADP, 7 mM G6P, and 0.4 U G6P dehydrogenase ml^{-1}) at 37°C. Microsomes were pre-incubated for 5 min prior to the addition of Na_2CrO_4 to a final concentration of 19.6 μM. Net enzymatic rates were obtained by subtracting the slow rates (the result of a slow chemical reduction by NADPH (10)) that were observed in the presence of pre-boiled microsomes (which were previously shown to lack enzymatic activity (2)). The reduction of Cr(VI) was stopped by the addition of 125 μl 2 M Na_2CO_3 (per 2.5 ml reaction volume) (10). The concentration of remaining Cr(VI) was measured colorimetrically by 1,5-diphenyl-carbazide (DPC) in acid solution (pH 2) (11) after removal of interfering reducing material by a charcoal/aluminum oxide mixture (12). Cr(VI) concentrations were determined from a standard curve with Na_2CrO_4 as the standard by measuring absorbance at 540 nm against a blank in which Na_2CrO_4 was omitted. Net enzymatic rates were obtained by subtracting the slow rates (the result of a slow chemical reduction by NADPH) that were observed in the presence of pre-boiled microsomes or proteoliposomes (which were previously shown to lack enzymatic activity). In some experiments, the NADPH-dependence of the reduction of Cr(VI) was assessed by removing individual components of the NADPH-generating system. In some experiments, NADH or NADPH (final concentration of 2 mM) was added from a 10 mM stock solution, instead of the NADPH-generating system. FeADP or $FeCl_3$ (final concentration = 12.4 µM) and/or deferoxamine (final concentration = 0.1 mM) were added prior to the 5 min preincubation.

Fe(III) Reduction Assay. Ferric reductase activity was assayed under anaerobic conditions by monitoring the production of Fe(II) over time as adapted from Myers and Myers (13). All solutions were pre-incubated in the anaerobic chamber for at least one hour before use. The assays were performed at 37°C in temperature-controlled stirred anaerobic cuvettes using an Aminco anaerobic cell accessory (SLM Instruments, Urbana, IL); the assay mix (3.0 ml total volume) consisted of: 150 mM KCl/25 mM HEPES (pH 7.35), 0.4 mM ferrozine, 0.03 mM Fe (either FeADP, ferric diethylenetriaminepentaacetic acid (FeDETPA), $FeCl_3$ or ferric citrate), and microsomal enzymes as specified. The anaerobic cuvettes were set up in an anaerobic chamber in a 37°C heating block, and sealed before removal from the chamber to maintain anaerobic conditions. The assay was started by the addition of 60 µl 50 mM anaerobic NAD(P)H to the sample cuvette (using the sealed plunger device of the anaerobic cell accessory); the reference cuvette received 60 µl anaerobic water at the same time. Activity was followed by an increase in absorbance over 3 min at 562 nm. Rates of Fe(III) reduction in the absence of enzymes, which were minimal, were subtracted from enzymatic rates to obtain the net values due to enzymatic activity. Using the extinction coefficient for the ferrozine-Fe(II) complex of 28 mM^{-1} cm^{-1} (14), changes in absorbance were converted to nmol of ferrozine-Fe(II) complex formed (equivalent to nmol Fe(III) reduced) per min at 37°C.

Ferric citrate, FeDETPA and $FeCl_3$ were purchased and made up as 10 mM stock solutions. FeADP was prepared by mixing 2 ml of 100 mM ferric nitrate with 6 ml of 100 mM ADP, and then adjusting the pH to 7.2 with $NaHCO_3$, and the final volume to 100 ml (15).

Effects of Enzyme Inhibitors. For experiments involving the use of specific enzyme inhibitors, the inhibitor was added during pre-incubation period and was therefore exposed to the enzyme prior to the initiation of the asay. The inhibitor stock solutions were made just before use.

Miscellaneous Procedures. Protein concentrations were determined by the Lowry method (16) modified as described to remove potential interference from detergents (17), with bovine serum albumin as the standard.

Antisera against purified recombinant human cytochrome b_5, P450 reductase, and b_5 reductase were raised in specific pathogen-free New Zealand white rabbits as described (5). IgG was

purified from the rabbit sera by ammonium sulfate precipitation and anion exchange chromatography (18). SDS-PAGE (8) and Western blot analysis (19) were done as previously described.

Cytochrome b_5 was determined by reduced-minus-oxidized difference spectra (20) using an Aminco DW-2000 spectrophotometer (SLM-Aminco Instruments, Urbana, IL); for liposomes containing cytochrome b_5 plus P450 reductase, NADPH was added to the sample cuvette and repetitive scans were done every minute (400–600 nm) until maximal reduction has occurred; dithionite was then added to the sample cuvette to ensure complete reduction of b_5, and the scan was repeated; the differences in absorbance (556–575 nm, $\varepsilon = 21\ mM^{-1}\ cm^{-1}$ (20, 21); and 426–409 nm, $\varepsilon = 185\ mM^{-1}\ cm^{-1}$ (21)) were used to calculate b_5 concentration.

An anaerobic chamber (5–6% H_2/balance N_2) was used to attain 0% O_2; Cr(VI) reduction rates under these conditions are indistinguishable from those in which the vials were made anaerobic by flushing with O_2-free N_2. (2). Solutions were pre-conditioned in small volumes in the anaerobic chamber for at least 1 hr. Aerobic conditions (21% O_2) were be achieved using open vials under room air while shaking at ~100 rpm.

Determination of Enzyme Activities. FMO3 activity was assessed as oxidation of methyl *p*-tolyl sulfide using a method adapted from Brunelle et al. (22). Specifically, the reaction was conducted in 50 mM potassium phosphate (pH 8.4) containing an NADPH-generating system (0.5 mM NADP, 2.0 mM G6P, 2 U G6P dehydrogenase ml^{-1}), 0.8 mM DETAPAC, 1 μM FAD. The FMO3 source was added and incubated with slow shaking for 2 min at 37°C. Methyl *p*-tolyl sulfide was added to a final concentration of 0.5 mM, and incubation was for 40 min at 37°C. Final total reaction volume was 0.25 ml. The reaction was stopped by adding 0.70 ml ice-cold acetonitrile, after which the tube was briefly vortexed and immediately iced. 20 mg NaCl was added to each tube, after which the tube was extensively vortexed. Following a 10 min centrifugation at 2000 x g at 4°C to separate the phases, the organic layer was analyzed by isocratic HPLC (mobile phase: 50% acetonitrile in water, 1.0 ml/min; Alltech C8 RSIL column, 250 x 4.6 mm; UV detection at 220 nm). The substrate (methyl *p*-tolyl sulfide) and product (methyl *p*-tolyl sulfoxide) were quantified by comparison of peak areas relative to those of standards of these two compounds.

NADH:b_5 reductase activity was determined as NADH-dependent ferricyanide reduction as described (6, 23).

NADPH:P450 reductase activity was measured at 37°C as cytochrome *c* reduction (24); the change in absorbance at 550 nm was followed over time in a 1-ml volume containing 0.1 μmol NADPH, 34 nmol cytochrome *c* "type III", 50 mM potassium phosphate (pH 7.7), 1 mM cyanide, and 0.1 to 0.5 mg microsomal protein; the rate of cytochrome *c* reduction was calculated based on the extinction coefficient ($\varepsilon = 29.5\ mM^{-1}\ cm^{-1}$ for reduced cytochrome *c*).

Goal #2 — Role of Human Microsomal Enzymes in Cr(VI) and Fe(III) Reduction:

Cr(VI) Reductase Activity in Subfractions of Human Microsomes. Human liver microsomes were subjected to isopycnic sucrose gradient ultracentrifugation. Fractions were collected and analyzed for NADH and NADPH-dependent Cr(VI) reduction activity, and for distribution of key proteins (cytochrome b_5, b_5 reductase and P450 reductase) by Western blot analysis. Figure 1A shows that the fractions which contained both cytochrome P450 reductase and cytochrome b_5 cumulatively accounted for 88.8% of the total NADPH-dependent Cr(VI) reduction activity. Likewise, the sucrose gradient fractions which contained both cytochrome b_5 and cytochrome b_5 reductase cumulatively accounted for 71.9% of the total NADH-dependent Cr(VI) reduction activity (Fig. 1B). The fractions which contained detectable levels of P450 reductase or cytochrome b_5 reductase, but not cytochrome b_5, showed minimal Cr(VI) reduction activity. Together, these results support a potential role for cytochrome b_5 in Cr(VI) reduction. However, cytochrome b_5 cannot directly receive reducing equivalents from NADPH or NADH. Therefore, b_5 reductase or P450 reductase are required to transfer reducing equivalents from NADH or NADPH

to cytochrome b_5, respectively. Overall, these results are consistent with a central role for cytochrome b_5 in both NADH- and NADPH-dependent Cr(VI) reduction in humans.

Generation and Characterization of Reconstituted Proteoliposomes. Since P450 reductase does not interact with cytochrome b_5 in solution, it was necessary to incorporate these proteins into artificial membranes. Attempts using a sonication method allowed for only limited interaction of the proteins, i.e. only about 11% of the total b_5 was reduced by P450 reductase. A reverse-phase evaporation liposome reconstitution procedure similarly provided only limited interaction of these proteins, i.e. only about 16–22% of the total b_5 was reduced by P450 reductase. Reconstituted proteoliposomes were successfully prepared by cholate dialysis. To determine the ratio that provided optimal interaction between the two proteins, numerous lipid:protein ratios ranging from 15:1 up to 119:1 were tried along with b_5:P450 reductase ratios from 1:1 up to 100:1. Complete interaction was obtained using a 250:1.1:1 molar ratio of egg yolk phosphatidylcholine:cytochrome b_5:P450 reductase. The interaction of P450 reductase with cytochrome b_5 in vesicle preparations was assessed by following the NADPH-dependent reduction of cytochrome b_5 in proteoliposomes containing P450 reductase only, cytochrome b_5 only or both proteins. The intensities of the alpha and Soret peaks were used to assess the extent of NADPH-dependent reduction of cytochrome b_5. Sodium dithionite was then added in excess to completely reduce all of the cytochrome b_5. The difference between the dithionite- and NADPH-reduced spectrum would account for any cytochrome b_5 that was not reduced by NADPH:P450 reductase. Comparison of these spectra showed that the electron transfer between cytochrome P450 reductase and cytochrome b_5 in proteoliposomes was efficient and complete (Fig. 2). Figure 2 also shows representative scans of proteoliposomes containing either cytochrome b_5 or cytochrome P450 reductase alone. These controls showed no direct reduction of cytochrome b_5 by NADPH. Together, these results demonstrate that cytochrome b_5 cannot directly accept reducing equivalents from NADPH, and that b_5 efficiently accepts electrons from NADPH:P450 reductase in these proteoliposomes.

Reduction of Cr(VI) by Reconstituted Proteoliposomes . Using this reconstituted proteoliposome system, the ability of cytochrome b_5 plus P450 reductase to mediate Cr(VI) reduction was determined. Proteoliposomes containing cytochrome b_5 alone showed no Cr(VI) reduction. Minimal Cr(VI) reduction was seen in proteoliposomes which contained only P450 reductase; this is consistent with previous findings that P450 reductase has poor Cr(VI)-reducing ability on its own (25). However, proteoliposomes containing cytochrome b_5 plus P450 reductase could reduce Cr(VI) at high rates. When the NADPH-dependent Cr(VI) reduction rates by proteoliposomes and human liver microsomes were normalized for cytochrome b_5 content, the NADPH-dependent Cr(VI) reduction rate mediated by human microsomes (0.605 ± 0.076 nmol min^{-1}) was essentially identical to that for proteoliposomes containing cytochrome b_5 plus P450 reductase (0.621 ± 0.139 nmol min^{-1}) (Fig. 3). These results clearly demonstrate that proteoliposomes containing cytochrome b_5 in combination with P450 reductase can account for essentially all the NADPH-dependent Cr(VI) reduction seen with human microsomes. No additional proteins are necessary to catalyze the NADPH-dependent reduction of Cr(VI) at rates consistent with those of human liver microsomes.

Fe(III) Stimulates Cr(VI) Reduction by Reconstituted Proteoliposomes. Since iron can stimulate the rate of Cr(VI) reduction by human microsomes (3), the ability of iron to stimulate Cr(VI) reduction by proteoliposomes was examined. A concentration-dependent effect was seen in which the addition of 12.4 μM of $FeCl_3$ produced the maximal stimulation of Cr(VI) reduction by proteoliposomes containing cytochrome b_5 and P450 reductase. Figure 4 shows results for proteoliposomes which were tested in the presence or absence of $FeCl_3$ (12.4 μM) and/or deferoxamine (0.1 mM), a specific Fe(III) chelator. Proteoliposomes had a NADPH-dependent Cr(VI) reduction rate of 0.30 ± 0.032 nmol min^{-1}. The Cr(VI) reduction rate was not significantly different (0.276 ± 0.14 nmol min^{-1}) when 0.1 mM deferoxamine was added, which indicated that there was minimal iron contamination in the proteoliposome preparation. Addition of exogenous $FeCl_3$ (12.4 μM) significantly stimulated the Cr(VI) reduction rate to 0.523 ± 0.028 nmol min^{-1}. The addition of deferoxamine abolished the stimulation by $FeCl_3$. Equivalent results

were obtained when FeADP was substituted for $FeCl_3$ in these experiments (not shown). These results are analogous to those previously reported for human microsomes (3).

Fe(III) and Quinone Reduction by Reconstituted Proteoliposomes. The ability of proteoliposomes to reduce Fe(III) was examined. Previous studies (3) had shown that Fe serves a catalytic role in human microsomal Cr(VI) reduction. Therefore, the rate of NADPH-dependent Fe(III) reduction should be equal to or greater than the iron-stimulated Cr(VI) reduction rate. Proteoliposomes containing cytochrome b_5 plus P450 reductase were able to reduce four different Fe(III) complexes ($FeCl_3$, Fe citrate, FeDETPA, and FeADP) at various rates (Fig. 5). These Fe(III) reduction rates were sufficient to account for the increased Cr(VI) reduction rates seen with the addition of FeADP or $FeCl_3$.

The reduction of quinones by human microsomes can also markedly stimulate Cr(VI) reduction rates (25). To assess the potential interactions of P450 reductase and cytochrome b_5 in proteoliposomes in mediating quinone reduction, quinone-mediated NADPH oxidation assays were performed. No oxidation of NADPH occurred when quinones were added to proteoliposomes which only contained cytochrome b_5. The addition of quinones to proteoliposomes which only contained cytochrome P450 reductase stimulated NADPH oxidation. However, the quinone-stimulated NADPH oxidation rate was further enhanced with proteoliposomes containing both cytochrome b_5 and cytochrome P450 reductase, such that the rate with both proteins was greater than the sum of the rates with either protein alone. These results demonstrated the cooperation of P450 reductase and cytochrome b_5 in quinone reduction.

Reconstituted Liposomes Generate Cr(V) During Cr(VI) Reduction. Since the reduction of Cr(VI) to Cr(III) is a 3-electron process, and there are no known single-step 3-electron donors in biological systems, Cr(V) and/or Cr(IV) are likely intermediates. Cr(V) has a distinct electron spin resonance (ESR) spectrum (g = 1.979) which can be examined semi-quantitatively by comparison of changes in the signal intensity over time (26). Human liver and lung microsomes generate Cr(V) during Cr(VI) reduction (4). Consistent with the predicted ability of cytochrome b_5 to transfer one electron at a time, Cr(V) was generated as a transient intermediate during Cr(VI) reduction by proteoliposomes containing cytochrome b_5 and P450 reductase (Figs. 6, 7).

Fe(III) Stimulates Cr(V) Reduction by Reconstituted Liposomes. The addition of exogenous iron stimulated not only the reduction of Cr(VI), but also the disappearance of Cr(V) (Fig. 7B). Fe(II) can directly reduce Cr(VI) (3), and the data suggest that Fe(II) might also directly reduce Cr(V), facilitating its more rapid disappearance. As a result, it appears that the repeated redox cycling of a small amount of Fe could facilitate the reduction of both Cr(VI) and Cr(V). This could hasten the formation of Cr(IV), a highly reactive Cr intermediate. These observations are similar to those observed with human liver and lung microsomes.

Generation of Hydroxyl Radical During Cr(VI) Reduction. Experiments were done to determine if hydroxyl radical (•OH) is generated as a consequence of Cr(VI) reduction by human microsomal enzymes. DEPMPO (5-Diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide; Oxis Research) was used as the spin trap because, unlike DMPO, it can trap and stabilize both •OH as DEPMPO/OH and superoxide radical anion (O_2^-) as DEPMPO/OOH, giving ESR spectra characteristic of each (27). DEPMPO is therefore able to distinguish between superoxide-dependent and -independent mechanisms that lead to formation of the hydroxyl adduct. Also, the DEPMPO/OOH adduct is 15 times more persistent than the DMPO/OOH spin adduct (27), and DEPMPO/OOH does not spontaneously decay to DEPMPO/OH (28).

During NADPH-dependent Cr(VI) reduction by proteoliposomes under aerobic conditions, an 8-line ESR spectrum (intensity ratio of 1:2:2:1:1:2:2:1) which is consistent with the DEPMPO/OH adduct (a^P = 47.3 G, a^H = 13.2 G, a^N = 14.0G) (29, 30) was detected (Fig. 8A). This signal was enhanced by the addition of H_2O_2 (Fig. 8F) and abolished by the addition of catalase (Fig. 8D). The Cr(V) signal can be seen simultaneously superimposed on the high-field end of the 8-line spectrum for DEPMPO/OH (Fig. 8A). Both the Cr(V) and DEPMPO/OH spectra were absent if either Cr(VI) or $NADP^+$ were omitted from the reaction mix (Fig. 8B,C), demonstrating that •OH was formed as a consequence of enzymatic Cr(VI) reduction. When Cr(VI) reduction was

conducted under anaerobic conditions, the Cr(V) signal was readily apparent but the DEPMPO/OH spectrum was absent (not shown). In the presence of excess formate, the DEPMPO/OH signal was largely replaced with that of DEPMPO/$^{\bullet}CO_2^-$ (DEPMPO/carbon dioxide radical anion) adduct (Fig. 8E). The increased signal intensity is attributed to an increased stability of the DEPMPO/$^{\bullet}CO_2^-$ adduct (see Fig. 8A and 8E). The results were analogous when human microsomes were substituted for proteoliposomes (not shown). The overall data are consistent with $^{\bullet}OH$ generation as a result of Fenton-type chemistry between H_2O_2 and Cr(V) and/or Cr(IV). The addition of deferoxamine to proteoliposomes did not decrease the Cr(VI) reduction rates (Fig. 4) (5), suggesting that Fe contamination is negligible in these experiments. The results also indicate that the proteoliposomes generate sufficient H_2O_2 to support $^{\bullet}OH$ formation.

The Central Role of Cytochrome b_5 in Cr(VI) Reduction. The sum total of evidence to date suggests that cytochrome b_5 is able to directly reduce Cr(VI) (Fig. 9A). It requires a suitable reductase (e.g. P450 reductase) to provide it with electrons originally derived from NADPH. The evidence also suggests that b_5 is able to reduce Fe(II), and Fe(II) acts as a reductant of Cr(VI) (Fig. 9B); the redox cycling of a small amount of Fe by cytochrome b_5 can result in the reduction of a large amount of Cr(VI). Preliminary studies are also consistent with a role for cytochrome b_5 in the reduction of certain quinones, which also have important implications for stimulation of Cr(VI) reduction (4, 25).

Methods Used to Accomplish Goal #2

Human Tissue. Human liver and lung tissues were provided by the Organ Transplant Unit at Froedtert Memorial Lutheran Hospital (Milwaukee, WI), with the approval of the Human Research Review Committee of the Medical College of Wisconsin. In all cases, the organs were removed from brain-dead organ transplant donors by the Organ Transplant Unit within approximately 30 min after death. The tissue was immediately iced, cut into small pieces, flash frozen in liquid nitrogen, and stored at –80°C as previously described (2). A partial characterization of the P450 isozyme contents of the hepatic microsomes from four of these subjects has been published (31, 32). Microsomal fractions were prepared from human tissue using a conventional differential centrifugation procedure as previously described (2).

Density Gradient Analysis of Human Microsomes. Human liver microsomes were subjected to isopycnic sucrose gradient ultracentrifugation as described (33). Human microsomes (100 µl) were mixed with 900 µl of sucrose (67%, w/w) in 10 mM PIPES-KOH pH 7.2 with 110 mM KCl. One ml aliquots of 50, 40, 30, 20, 10, 5, 2.5, and 0% sucrose (w/w) in buffer were successively layered on top of this suspension. These gradients were centrifuged at 25,000 rpm for 16 hours at 4°C in a Beckman SW28.1 rotor. Fractions (0.7 ml) were then collected and analyzed for Cr(VI) reduction activity, and for relative content of key proteins (cytochrome b_5, cytochrome b_5 reductase and cytochrome P450 reductase) by Western blot analysis.

Liposome Preparation and Reconstitution. Human recombinant P450 reductase and cytochrome b_5 were purchased from Panvera Corp. (Madison, WI). Liposomes containing P450 reductase alone, cytochrome b_5 alone, or P450 reductase plus cytochrome b_5 were reconstituted with minor modifications to the procedure described by Ingelman-Sundberg and Glaumann (34). Briefly, the liposomes were prepared from egg yolk phosphatidylcholine. The phospholipids (10 mg) in chloroform were placed in a 13 mm glass test tube in an anaerobic chamber and kept on ice. The chloroform was then evaporated under a stream of N_2 to absolute dryness at room temperature. The lipids were resuspended in 1.778 ml of 10 mM Tris-HCl buffer, pH 7.2, containing 50 mM NaCl and 0.2 mM EDTA. Sodium cholate (10%, w/v; Calbiochem, San Diego, CA) in distilled water was added to the lipid suspension to a final concentration of 1.0%. The phospholipid suspension was then rocked at 4°C for one hr until the solution cleared. The proteins were added to the liposomes at a molar ratio of 250:1.1:1 (lipid:b_5:P450 reductase). Cytochrome b_5 was added first and rocked at 4°C for 2 hr prior to the addition of P450 reductase which was then rocked for one additional hr at 4°C. The samples were then dialyzed for 6 hr (changing the buffer every 2 hr) in Slide-A-Lyzer mini dialysis units (Pierce Inc., Rockford, IL; 10,000 MWCO) in 250 ml of 10 mM Tris-HCl pH 7.2 containing 50 mM NaCl, 0.2 mM EDTA and 1 mM PMSF.

Cytochrome b_5 Spectral Analysis. The reduction of cytochrome b_5 was followed spectrophotometrically using an Aminco DW2000 (SLM Instruments). Proteoliposomes containing P450 reductase only, cytochrome b_5 only and P450 reductase plus cytochrome b_5 were diluted to a concentration of 14.4 µg protein in 2 ml of 0.1 M potassium phosphate buffer, pH 7.0. The sample was vortexed for 10 sec to completely oxidize all cytochrome b_5, and was then divided equally between sample and reference cuvets. The sample was then scanned once from 400 to 600 nm to establish the baseline. NADPH (0.2 mM) was added to the sample cuvette, it was mixed by inversion and then scanned four times (2 min per scan). Excess dithionite was then added to the sample cuvette, mixed by inversion and then rescanned. The maximal difference in absorbance (peak minus trough) of the alpha and Soret peaks was used to calculate the concentration of cytochrome b_5 using extinction coefficients of 21 mM^{-1} cm^{-1} and 185 mM^{-1} cm^{-1} for the alpha and Soret peaks, respectively (20, 35).

ESR Spectroscopy for Cr(V). Cr(V), a d^1 paramagnetic species, has a distinct EPR spectrum at conventional X-band frequency that consists of a sharp line at $g = 1.98$ (26, 36). Relative changes in the levels of Cr(V) over time were estimated by comparison of changes in the signal intensity (26). Cr(VI) reduction experiments were established as described above except that the total volume of the assay was reduced to 1.25 ml and the initial Cr(VI) level was 100 µM. Aliquots (0.3 ml) of the Cr(VI) reduction assays were taken at periodic intervals and frozen in quartz tubes by immersion in liquid nitrogen (77 K). The samples were stored at 77 K for up to one week until analyzed by ESR; we have demonstrated that Cr(V) is stable for at least several months at 77 K. The samples in the quartz tubes were placed in a finger Dewar with liquid nitrogen, and the ESR spectra were recorded using a Varian Century Series Spectrometer, which includes a Gauss meter for magnetic-field calibration and a frequency counter. Instrument settings were as follows: 5.0 G modulation amplitude, 2×10^4 receiver gain, 0.128 s time constant, 8.996 GHz microwave frequency, sweep width = 400 G, field set = 3300 G, modulation frequency = 100 kHz, scan time = 2 min. Samples for Cr(V) were initially analyzed at two levels of microwave power, 16 and 34 dB; while signal intensity was larger at 16 dB, relative changes in signal intensity were very similar for the two microwave powers. Data are shown at 34 dB because signals did not reach saturation at this power. Samples for Cr(V) were initially analyzed with two field modulations (5 G and 1 G); similar results were obtained with either modulation. Relative levels of Cr(V) were measured directly from the size of EPR signals as determined by $I\Delta H^2$ (I = peak-to-trough intensity in cm normalized to a constant receiver gain; ΔH = line width in Gauss between peak minimum and maximum of the first derivative spectrum). Representative scans were repeated to verify reproducibility.

Fe(III) Reduction Assay. The details are the same as described for goal #1.

Cr(VI) Reduction Assay. The details are the same as described for goal #1.

Miscellaneous Procedures. The details are the same as described for goal #1.

Contribution to Student Training

In addition to generating additional research findings, a goal of this AASERT award was to provide the graduate student with training in molecular toxicology as it applied to an AFOSR project on the mechanisms of Cr(VI) reduction. Through these research accomplishments, the student has learned and mastered several valuable techniques relevant to various disciplines. The sum total of techniques mastered to date include: preparation of human microsomes, assays for microsomal proteins (e.g. b_5 reductase, P450 reductase, cytochrome b_5), reduction of Cr(VI) and Fe(III), NAD(P)H-dependent quinone reduction, PCR, PCR primer design, use of restriction enzymes, agarose gel electrophoresis, cloning, DNA sequencing, plasmid minipreps, selection and use of fusion vectors for bacterial expression, affinity chromatography, ion exchange chromatography, immunoaffinity chromatography, use of detergents for solubilizing hydrophobic proteins, Lowry protein assay, SDS-PAGE, Western blotting, anaerobic techniques, liposome preparation/characterization/reconstitution, isopycnic gradient ultracentrifugation, phospholipid

extraction and lipid phosphorus analysis, ESR for Cr(V), and ESR spin trapping for reactive oxygen species. These will be highly valuable to him in his future career as a research toxicologist.

NEW DISCOVERIES, CUMULATIVE:

1. Various tools were generated for use in discerning the mechanism of human microsomal Cr(VI) reduction:
   a. Bacterial expression system approaches were developed and implemented for the following human microsomal enzymes: FMO3, P450 reductase, and cytochrome b_5 reductase.
   b. Polyclonal antibodies against recombinant FMO3-MBP, cytochrome b_5, and P450 reductase were generated.
2. Flavin-containing monooxygenase isoform 3 (FMO3), the major hepatic FMO in humans, does not have an apparent significant role in microsomal Cr(VI) or Fe(III) reduction.
3. By itself, human cytochrome P450 reductase is a relatively poor reducer of Cr(VI) and Fe(III). Since it cannot accept electrons directly from NAD(P)H, cytochrome b_5 is also unable to mediate Cr(VI) and Fe(III) reduction on its own.
4. Cytochrome b_5 reductase does not appear to directly mediate Cr(VI) or Fe(III) reduction at significant rates, but could play a significant role by acting together with cytochrome b_5.
5. The addition of cytochrome b_5 plus b_5 reductase to human microsomes significantly increased NADH-dependent Cr(VI) reduction rates, suggesting that b_5 reductase likely acts together with cytochrome b_5 to mediate Cr(VI) reduction; these proteins do not interact in solution, but require a lipid environment to facilitate electron flow from b_5 reductase to cytochrome b_5.
6. Density gradient separation of human microsomes showed that the vast majority of NAD(P)H-dependent Cr(VI) reduction was confined to those fractions in which cytochrome b_5, P450 reductase, and b_5 reductase were in common. This supports our hypothesis that these are the key players in Cr(VI) reduction.
7. Human liver or lung do not represent practical sources for obtaining large amounts of purified cytochrome b_5 reductase.
8. The following tools were generated for use in discerning the mechanism of human microsomal Cr(VI) reduction:
   a. A new bacterial expression system approach was developed for human b_5 reductase, which results in more stable activity after extraction from *E. coli*.
   b. Polyclonal antibodies were generated against two human b_5 reductase fusions. While both recognized b_5 reductase in Western blots, attempts to use them for immunopurification were unsuccessful. They could inhibit ferricyanide reductase activity of the b_5 reductase-CBP fusion, but were unable to block electron transfer to cytochrome b_5 in microsomes or the NADH-dependent Cr(VI) reduction by microsomes.
9. A reconstituted liposome approach was modified and optimized which allowed for rapid and complete electron transfer from P450 reductase to cytochrome b_5. When normalized to b_5 content, these liposomes reduce Cr(VI) at rates comparable to those of human microsomes. This demonstrates conclusively that the interaction of P450 reductase with cytochrome b_5 accounts for essentially all of the NADPH-dependent Cr(VI)-reducing activity of human microsomes.
10. Trace amounts of Fe(III) stimulate Cr(VI) reduction by liposomes containing P450 reductase to cytochrome b_5. This stimulation is abolished by the iron chelator deferoxamine. These results are analogous to those previously reported for human microsomes.
11. Liposomes containing P450 reductase to cytochrome b_5 reduce Fe(III) at rates sufficient to explain the ability of Fe(III) to stimulate Cr(VI) reduction. These results suggest that the interaction of P450 reductase with cytochrome b_5 is also responsible for the reduction of Fe(III) to Fe(II); Fe(II) is a known reductant of Cr(VI) (3).

    The addition of quinones to proteoliposomes containing both P450 reductase and cytochrome b_5 stimulated NADPH oxidation to a much greater extent than with

proteoliposomes containing either protein alone. Thus, P450 reductase and cytochrome b_5 cooperate to mediate quinone reduction, which likely accounts for the quinone-mediated stimulation of Cr(VI) reduction previously reported for human microsomes (25).

12. Liposomes containing P450 reductase and cytochrome b_5 generate Cr(V) as a transient intermediate during Cr(VI) reduction. Cr(V) is a reactive intermediate that is likely responsible for some of the toxicity associated with Cr(VI) exposure.
13. Trace amounts of Fe(III) also hasten the reduction of Cr(V) by liposomes containing both P450 reductase and cytochrome b_5. This suggests that Fe(II) can also reduce Cr(V). The repeated redox cycling of a small amount of Fe could facilitate the reduction of both Cr(VI) and Cr(V). This could hasten the formation of Cr(IV), a highly reactive Cr intermediate.
14. The reduction of Cr(VI) by human microsomes or by proteoliposomes containing both P450 reductase and cytochrome b_5 results in the generation of hydroxyl radical ($^{\bullet}OH$), a highly reactive species capable of damaging numerous cell components. The reduction of Cr(VI) by these microsomal enzymes could therefore result in pronounced oxidative damage to cells.
15. Significant levels of malonaldehyde, an indicator of lipid peroxidation, are generated during the reduction of Cr(VI) by human microsomes. This is consistent with the generation of $^{\bullet}OH$.
16. The following protocols were developed:
    a. A liposome reconstitution procedure which allows for efficient and complete interaction of purified human recombinant P450 reductase and cytochrome b_5.
    b. An HPLC method was devised using a polar-RP column to allow separation and detection of malonaldehyde without interference from NADH, NAD, Cr(VI), Fe(III), and endogenous microsomal components.

LISTING OF COMMUNICATIONS AND INTERACTIONS:

Personnel Involved (15 AUG 97 to 14 AUG 00):

Charles R. Myers, Ph.D., Principal Investigator. No salary support was provided to the P.I. from this award, but 10% effort was dedicated to mentor the research training of the graduate student. 15 AUG 97 to 14 AUG 00.

Paul Jannetto, B.S., Graduate Student. 100% effort and stipend — this award. 15 AUG 97 to 14 AUG 00.

Publications (15 AUG 97 to 14 AUG 00):

P. J. Jannetto, W. E. Antholine, and C. R. Myers. 2000. Cytochrome b_5 plays a key role in human microsomal chromium(VI) reduction. ***Toxicology***. Accepted for publication.

Meetings/Abstracts (15 AUG 97 to 14 AUG 00):

P. J. Jannetto, B. P. Carstens, J. M. Myers, and C. R. Myers. 1999. The reductive pathway of chromium(VI) in human microsomes. *ISSX Proceedings of the 9th North American ISSX Meeting*, abstract 83, p. 42.

P. J. Jannetto, W. E. Antholine, and C. R. Myers. 2000. Cytochrome b_5 plays a key role in human microsomal chromium (VI) reduction. *Abstr. 13th Ann. Sci. Meeting Great Lakes Chptr. Amer. Soc. Pharmacol. Exper.Ther.*, abstract S-5, p. 8.

P. J. Jannetto, W. E. Antholine, J. M. Myers, and C. R. Myers. 2000. Chromium (VI) reduction mediated by cytochrome b_5 in human hepatic microsomes leads to hydroxyl radical formation and oxidative damage. *Abstr. Soc. Forensic Toxicologists*, abstract P41.

Student Seminars and Presentations (15 AUG 97 to 14 AUG 00):

21 APR 98, Dept. of Pharmacology & Toxicology, Medical College of Wisconsin
Seminar presented by: Paul Jannetto
Title: "The Potential Role of Cytochrome b_5 Reductase in Chromium Reduction".
The seminar was attended by members of various departments including Pharmacology and Toxicology, Biochemistry, and others.

22–23 OCT 98, Medical College of Wisconsin, Graduate Student Research Forum.
Poster presented by: Paul J. Jannetto
Title: "Cloning, expression, and purification of human cytochrome b_5 reductase".
The poster session was attended by faculty, staff, and students of all basic science departments of the Medical College of Wisconsin, as well as by students and faculty from several Wisconsin colleges and universities.

25 MAY 99, Dept. of Pharmacology & Toxicology, Medical College of Wisconsin
Seminar presented by: Paul J. Jannetto
Title: "A role for NADH:cytochrome b_5 reductase in chromium reduction and toxicity".
The seminar was attended by members of various departments including Pharmacology and Toxicology, Biochemistry, and Biophysics.

7–8 OCT 99, Medical College of Wisconsin, Graduate Student Research Forum.
Poster presented by: Paul J. Jannetto, B. P. Carstens, J.M. Myers and C.R. Myers
Title: "The reductive pathway of Cr(VI) in human microsomes".
The poster session was attended by faculty, staff, and students of all basic science departments of the Medical College of Wisconsin, as well as by students and faculty from several Wisconsin colleges and universities.

29 FEB 00, Dept. of Pharmacology & Toxicology, Medical College of Wisconsin
Seminar presented by: Paul J. Jannetto
Title: "A common pathway for human microsomal Cr(VI) reduction mediated by cytochrome b_5".
The seminar was attended by members of various departments including Pharmacology and Toxicology, Biochemistry, and Biophysics.

Interactions (15 AUG 97 to 14 AUG 00):

A collaboration was established with David Petering, Ph.D., Professor of Chemistry at the University of Wisconsin–Milwaukee, who is an expert in metal-binding proteins, metal chemistry, and DNA damage by anticancer agents. Dr. Petering has developed a 13-mer double-stranded defined oligonucleotide which acts as a model for DNA damage by bleomycin, an anti-tumor agent. Dr. Petering provided us with this model DNA for preliminary experiments on the potential for DNA strand breaks as a result of reactive species generated during Cr(VI) reduction by human enzymes. Preliminary studies were negative, but methods have yet to be optimized for Cr(VI) reduction parameters. This model DNA may therefore still be of use to us in future studies on DNA damage.

Consultative and Advisory Functions (15 AUG 97 to 14 AUG 00):

None.

Technology Transfers (15 AUG 97 to 14 AUG 00):

The following technology transfers are directly related to research tools derived from this project:

1. Customer: Dr. Mary L. Haasch
   Chesapeake Biological Laboratory
   University of Maryland Center for Environmental Science
   One Williams Street
   Solomons, MD 20688-0038
   phone no.: 410-326-7238
   FAX: 410-326-7210
   email: haasch@cbl.umces.edu

   Result: Early in the project we developed an antipeptide antibody that specifically recognizes the major human hepatic flavin-containing monooxygenase (FMO3). Some of this antibody was transferred to Dr. Haasch.

   Application: Dr. Haasch will use the antibody for Western blots as a biomonitoring tool to study the induction of FMO in aquatic species.

2. Customer: Dr. David Petering
   Dept. of Chemistry
   University of Wisconsin–Milwaukee
   P.O. Box 413
   Milwaukee, WI 53201
   phone no.: 414-229-5853
   FAX: 414-229-5530
   email: petering@csd.uwm.edu

   Result: We modified and optimized a liposome reconstitution system that facilitates the rapid electron transfer between purified human recombinant P450 reductase and cytochrome b_5 in vitro. When normalized to b_5 content, this artificial system reduces Cr(VI) at rates comparable to those of human microsomes. This represents a powerful tool to study the role of these cooperative proteins without interference from the complexity of subcellular microsomal fractions. The development of this liposome system has been described in an accepted publication (5). Discussions were held in the spring of 2000 on the utility of this technology to Dr. Petering, and Dr. Petering's staff was trained in the liposome reconstitution procedure in AUG 00.

   Application: Dr. Petering will use the liposomes reconstituted with P450 reductase and cytochrome b_5 to study their ability to reductively activate bleomycin (an anticancer drug) to forms which are responsible for DNA damage. This research will contribute directly to a more complete understanding of the mechanism of action and toxicity of this drug.

Inventions (15 AUG 97 to 14 AUG 00):

No inventions.

Honors/Awards (15 AUG 97 to 14 AUG 00):

1. P.J. Jannetto was awarded First Prize for Outstanding Research Poster Presenter, 7–8 OCT 99, Medical College of Wisconsin, Graduate Student Research Forum. The competition included graduate students from all departments, including several more senior students.

2. P.J. Jannetto was awarded 1st Place in the Graduate Student Poster Competition at the 13th Annual Scientific Meeting of the Great Lakes Chapter of the American Society for Pharmacology and Experimental Therapeutics, 01 JUN 00, Midwestern University, Downers Grove, IL. The competition included graduate students from several institutions, including several more senior students.
3. P.J. Jannetto was a recipient of a Student Grant Award from the Society of Forensic Toxicologists; the award paid his registration fees, etc., for attendance and presentation at their annual meeting.

BIBLIOGRAPHY

1. Nakagawa, K., Matsubara, T., Kinoshita, I., Tsuchiya, E., Sugano, H., et al. 1984. Surveillance study of a group of chromate workers — early detection and high incidence of lung cancer (in Japanese). *Lung Cancer* 24:301–310

2. Pratt, P. F., Myers, C. R. 1993. Enzymatic reduction of chromium(VI) by human hepatic microsomes. *Carcinogenesis* 14:2051–2057

3. Myers, C. R., Myers, J. M. 1998. Iron stimulates the rate of reduction of hexavalent chromium by human microsomes. *Carcinogenesis* 19:1029–1038

4. Myers, C. R., Myers, J. M., Carstens, B. P., Antholine, W. E. 2000. Reduction of chromium(VI) to chromium(V) by human microsomal enzymes: effects of iron and quinones. *Toxic Substance Mechanisms* in press

5. Jannetto, P. J., Antholine, W. E., Myers, C. R. 2000. Cytochrome b_5 plays a key role in human microsomal chromium(VI) reduction. *Toxicology* in press

6. Yang, M.-X., Cederbaum, A. I. 1994. Fractionation of liver microsomes with polyethylene glycol and purification of NADH-cytochrome b_5 oxidoreductase and cytochrome b_5. *Archives of Biochemistry and Biophysics* 315:438–444

7. Myers, C. R., Myers, J. M. 1997. Cloning and sequence of *cymA*, a gene encoding a tetraheme cytochrome *c* required for reduction of iron(III), fumarate, and nitrate by *Shewanella putrefaciens* MR-1. *Journal of Bacteriology* 179:1143–1152

8. Myers, J. M., Myers, C. R. 1998. Isolation and sequence of *omcA*, a gene encoding a decaheme outer membrane cytochrome *c* of *Shewanella putrefaciens* MR-1, and detection of *omcA* homologs in other strains of *S. putrefaciens*. *Biochimica et Biophysica Acta* 1373:237–251

9. Myers, J. M., Myers, C. R. 2000. Role of the tetraheme cytochrome CymA in anaerobic electron transport in cells of *Shewanella putrefaciens* MR-1 with normal levels of menaquinone. *Journal of Bacteriology* 182:67–75

10. Mikalsen, A., Alexander, J., Andersen, R. A., Daae, H.-L. 1989. Reduction of hexavalent chromium in a reconstituted system of cytochrome P-450 and cytochrome b_5. *Chemico-Biological Interactions* 71:213–221

11. Sandell, E. B. 1959. *Colorimetric Determinations of Traces of Metals*. New York: Interscience

12. Ryberg, D. 1986. Problems with methods for measurement of hexavalent chromium applied to biological samples. *Acta Pharmacol. Toxicol.* 59, Suppl. VII:624–626

13. Myers, C. R., Myers, J. M. 1993. Ferric reductase is associated with the membranes of anaerobically grown *Shewanella putrefaciens* MR-1. *FEMS Microbiology Letters* 108:15–22

14. Carter, P. 1971. Spectrophotometric determination of serum iron at the submicrogram level with a new agent (ferrozine). *Analytical Biochemistry* 40:450–458

15. Asaumi, A., Ogino, T., Akiyama, T., Kawabata, T., Okada, S. 1996. Oxidative damages by iron-chelate complexes depend on the interaction with the target molecules. *Biochemistry and Molecular Biology International* 39:77–86

16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* 193:265–275

17. Dulley, J. R., Grieve, P. A. 1975. A simple technique for eliminating interference by detergents in the Lowry method of protein determination. *Analytical Biochemistry* 64:136–141

18. Myers, C. R., Collins, M. L. P. 1986. Cell-cycle-specific oscillation in the composition of chromatophore membrane in *Rhodospirillum rubrum*. *Journal of Bacteriology* 166:818–823

19. Myers, C. R., Porgilsson, B., Myers, J. M. 1997. Antibodies to a synthetic peptide that react with flavin-containing monooxygenase (HLFMO3) in human hepatic microsomes. *Journal of Pharmacological and Toxicological Methods* 37:61–66

20. Kajihara, T., Hagihara, B. 1968. Crystalline cytochrome b_5. I. Purification of crystalline cytochromes b_5 from rabbit liver. *Journal of Biochemistry (Tokyo)* 63:453–461

21. Estabrook, R. W., Werringloer, J. 1978. The measurement of difference spectra: application to the cytochromes of microsomes. *Methods in Enzymology* 52, pt. C:212–220

22. Brunelle, A., Bi, Y.-A., Russell, B., Luy, L., Berkman, C., et al. 1997. Characterization of two human flavin-containing monooxygenase (form 3) enzymes expressed in *Escherichia coli* as maltose binding protein fusions. *Drug Metabolism & Disposition* 25:1001–1007

23. Watanabe, F., Nakano, Y., Saido, H., Tamura, Y., Yamanaka, H. 1992. Cytochrome b_5/cytochrome b_5 reductase complex in rat liver microsomes has NADH-linked aquacobalamin reductase activity. *Journal of Nutrition* 122:940–944

24. Williams, C. H., Kamin, H. 1962. Microsomal triphosphopyridine nucleotide-cytochrome *c* reductase of liver. *Journal of Biological Chemistry* 237:587–595

25. Myers, C. R., Porgilsson, B., Carstens, B. P., Myers, J. M. 1999. Naphthoquinones stimulate the rate of reduction of hexavalent chromium by human microsomes. *Toxic Substance Mechanisms* 18:103–128

26. Stearns, D. M., Wetterhahn, K. E. 1994. Reaction of chromium(VI) with ascorbate produces chromium(V), chromium(IV), and carbon-based radicals. *Chemical Research in Toxicology* 7:219–230

27. Frejaville, C., Karoui, H., Tuccio, B., Le Moigne, F., Culcasi, M., et al. 1995. 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide: a new efficient phosphorylated nitrone for the in vitro and in vivo spin trapping of oxygen-centered radicals. *Journal of Medicinal Chemistry* 38:258–265

28. Vásquez-Vivar, J., Hogg, N., Martásek, P., Karoui, H., Tordo, P., et al. 1999. Effect of redox-active drugs on superoxide generation from nitric oxide synthases: biological and toxicological implications. *Free Radical Research* 31:607–617

29. Singh, R. J., Karoui, H., Gunther, M. R., Beckman, J. S., Mason, R. P., et al. 1998. Reexamination of the mechanism of hydroxyl radical adducts formed from the reaction

between familial amyotrophic lateral sclerosis-associated Cu,Zn superoxide dismutase mutants and H_2O_2. *Proceedings of the National Academy of Sciences USA* 95:6675–6680

30. Karoui, H., Hogg, N., Fréjaville, C., Tordo, P., Kalyanaraman, B. 1996. Characterization of sulfur-centered radical intermediates formed during the oxidation of thiols and sulfite by peroxynitrite. *Journal of Biological Chemistry* 271:6000–6009

31. Wrighton, S. A., Vandenbranden, M., Becker, G. W., Black, S. D., Thomas, P. E. 1992. Two monoclonal antibodies recognizing different epitopes on rat cytochrome IIB1 react with human IIE1. *Molecular Pharmacology* 41:76–82

32. Cashman, J. R., Park, S. B., Yang, Z.-C., Wrighton, S. A., Jacob, P. I., et al. 1992. Metabolism of nicotine by human liver microsomes:stereoselective formation of *trans*-nicotine *N'*-oxide. *Chemical Research in Toxicology* 5:639–646

33. Rigaud, J. L., Bluzat, A., Buschlen, S. 1983. Incorporation of bacteriorhodopsin into large unilamellar liposomes by reverse phase evaporation. *Biochemical and Biophysical Research Communications* 111:373–382

34. Ingelman-Sundberg, M., Glaumann, H. 1980. Incorporation of purified components of the rabbit liver microsomal hydroxylase system into phospholipid vesicles. *Biochimica et Biophysica Acta* 599:417–435

35. Omura, T., Sato, R. 1964. The carbon monoxide binding pigment of liver microsomes. *Journal of Biological Chemistry* 239:2370–2378

36. Molyneux, M. J., Davies, M. J. 1995. Direct evidence for hydroxyl radical-induced damage to nucleic acids by chromium(VI)-derived species. *Carcinogenesis* 16:875–882

TABLES AND FIGURES

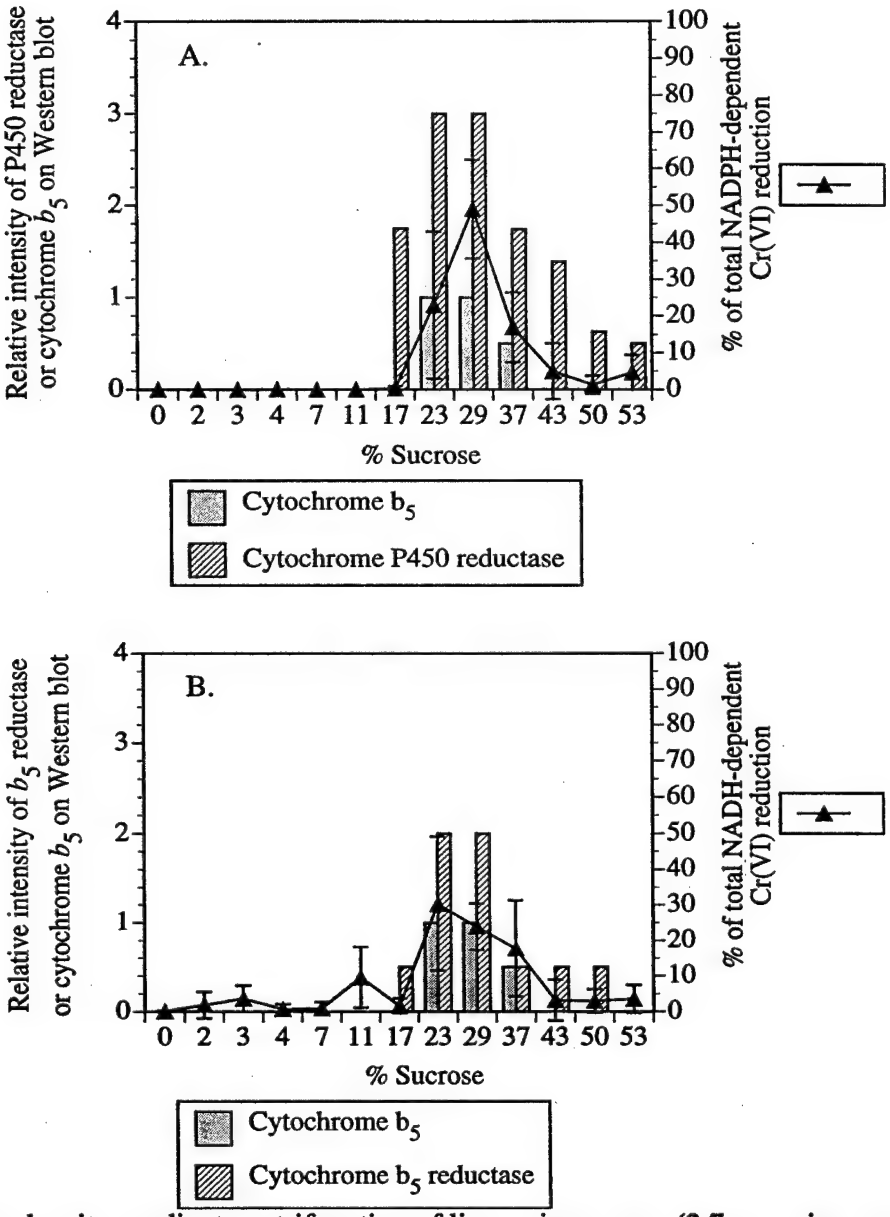


Fig. 1. Sucrose density gradient centrifugation of liver microsomes (2.7 mg microsomal protein). **(A):** distribution of NADPH-dependent Cr(VI) reduction (s) versus P450 reductase (vertical bars, stripped) and cytochrome b_5 (vertical bars, solid). **(B):** distribution of NADH-dependent Cr(VI) reduction (s) versus b_5 reductase (vertical bars, stripped) and cytochrome b_5 (vertical bars, solid). Each gradient was divided into thirteen successive fractions (700 µl each), top to bottom, and 200 µl analyzed for Cr(VI) reduction in a standard assay containing 20 µM Na_2CrO_4 in a total volume of 2.5 ml. Electron donors were either the NADPH-generating mix (panel A) or 2 mM NADH (panel B). The net enzymatic rates were obtained by subtracting the slow rates observed with pre-boiled fractions. The results (mean ± S.D., $n = 3$) are expressed as the % of total Cr(VI) reduction seen in each gradient. The location of key proteins was analyzed by Western blotting using polyclonal antibodies specific for the three proteins of interest. The intensities of the relevant Western blot bands (mean, $n = 4$) were visually semi-quantified from 0 (represents no band present) to 4 (represents maximal intensity).

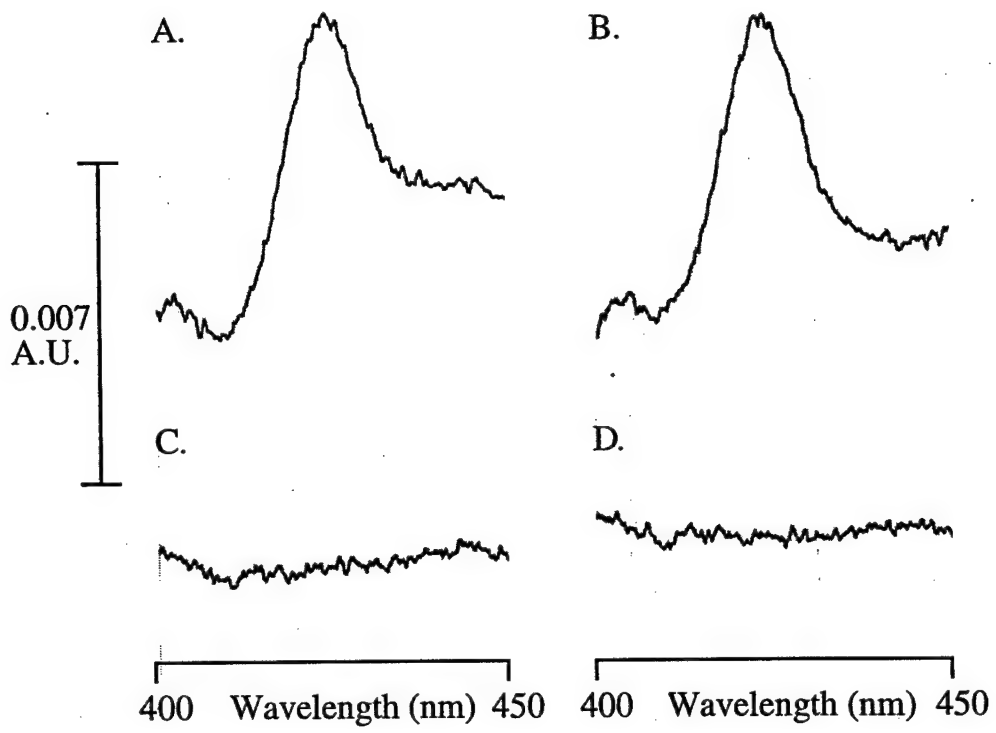


Fig. 2. Wavelength scans (400–450 nm) of reduced-minus-oxidized cytochrome b_5 in proteoliposomes. **(A):** Proteoliposomes containing cytochrome b_5 (0.039 nmol) and P450 reductase (0.035 nmol) after a 8-min incubation at 37°C with NADPH (1 mM). **(B):** Sample A to which an excess of sodium dithionite was added. **(C):** Same as A, except proteoliposomes contained only cytochrome b_5. **(D):** Same as A, except proteoliposomes contained only P450 reductase.

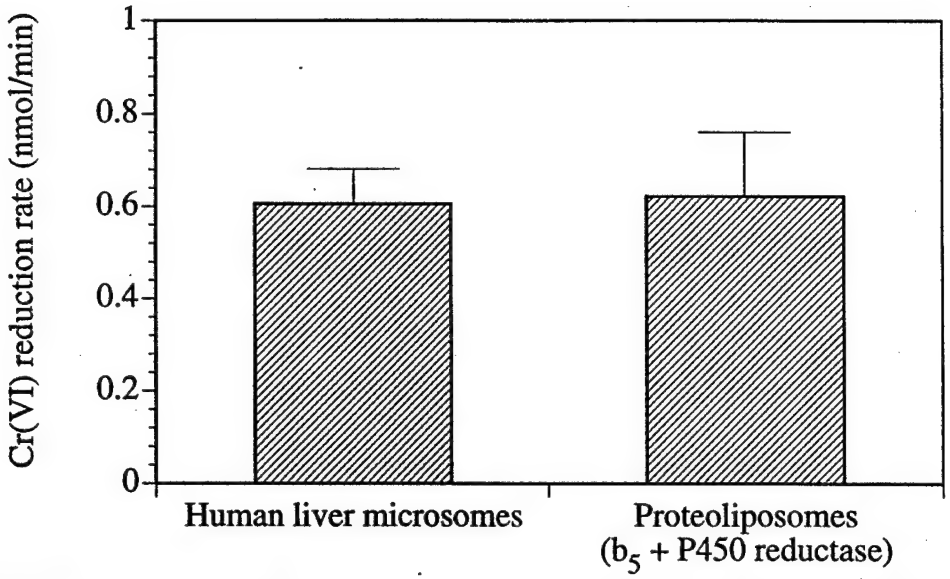


Fig. 3. NADPH-dependent Cr(VI) reduction rates catalyzed by proteoliposomes containing cytochrome b_5 and P450 reductase versus human liver microsomes. Results (mean ± S.D., $n = 3$) shown are normalized to 0.109 nmol of cytochrome b_5. No statistical difference between these rates was detected.

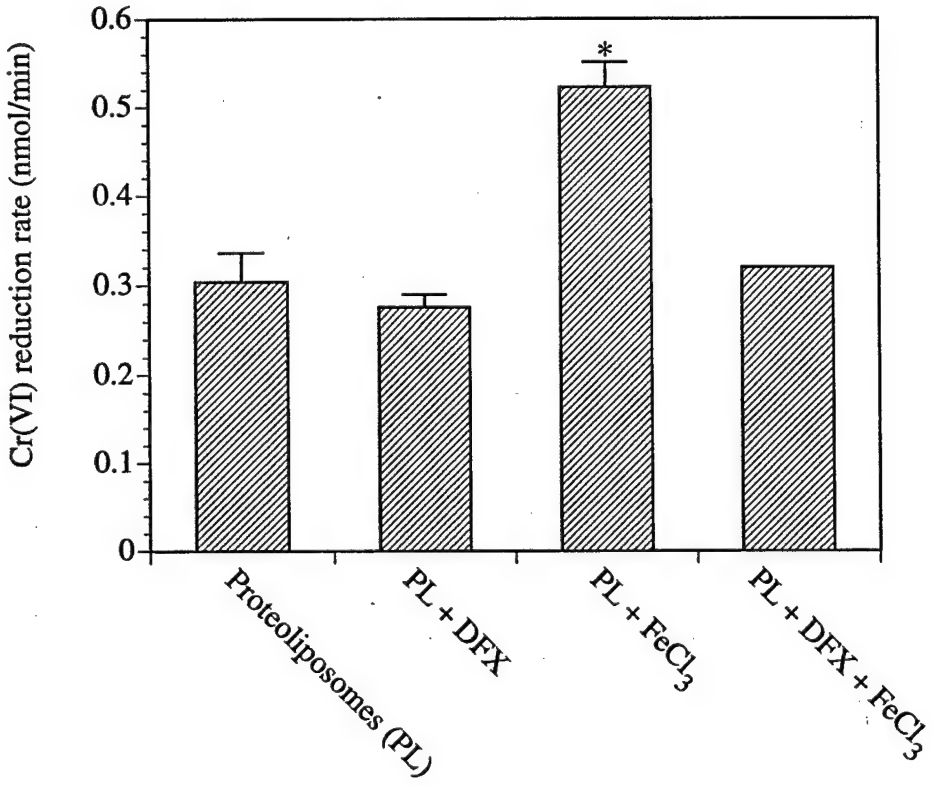


Fig. 4. Effect of the addition of $FeCl_3$ (12.4 μM) and/or 0.1 mM deferoxamine (DFX) on NADPH-dependent Cr(VI) reduction by proteoliposomes containing cytochrome b_5 (0.024 nmol) and P450 reductase (0.022 nmol). NADPH (2 mM) was the electron donor. Results shown represent the mean ± S.D., $n = 3$. (* $p < 0.01$)

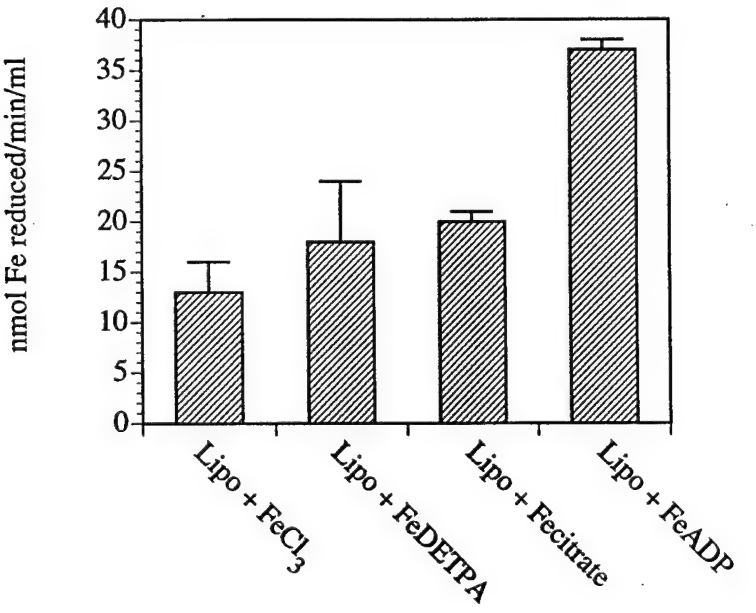


Fig. 5. Anaerobic Fe(III) reduction by proteoliposomes (Lipo) containing cytochrome b_5 (0.024 nmol) and P450 reductase (0.022 nmol). $FeCl_3$, FeDETPA, Fe citrate, and FeADP were added to achieve a final concentration of 12.4 μM. Results shown represent the mean ± S.D., $n = 3$.

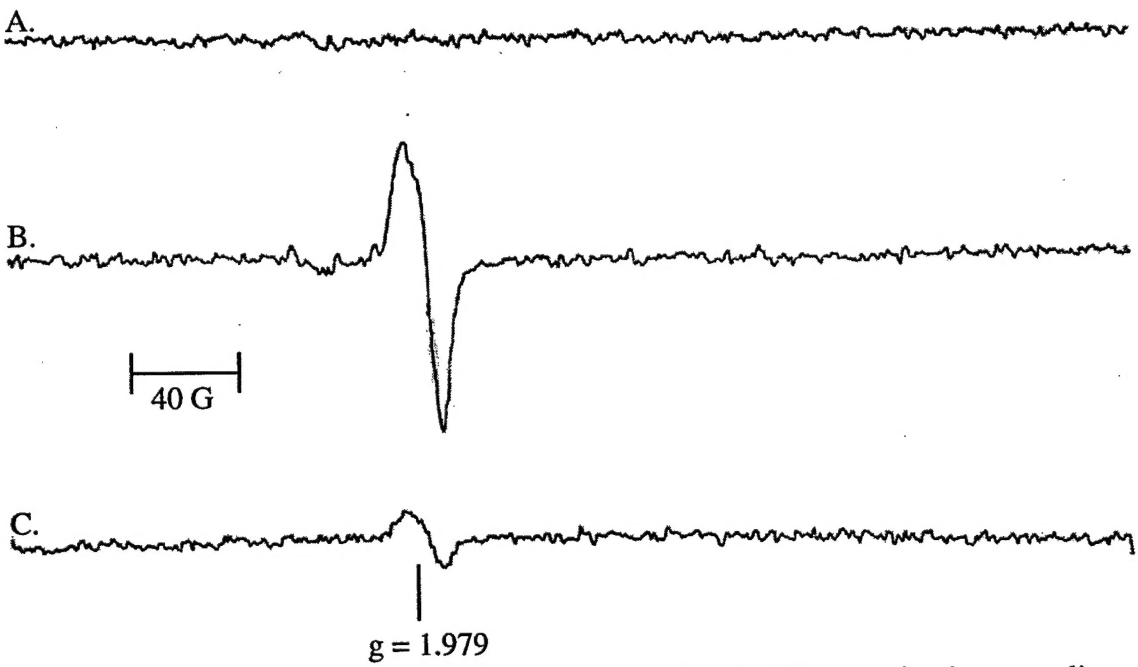


Fig 6. Characteristic EPR spectra of Cr(V) generated during Cr(VI) reduction by proteoliposomes containing cytochrome b_5 plus P450 reductase. Cr(VI) reduction experiments were done as described in the Methods section, except the total volume of the assay was 1.25 ml and the initial Cr(VI) level was 100 μM. Aliquots (0.3 ml) of the Cr(VI) reduction assays were taken at periodic time intervals (**A:** 0 min, **B:** 5 min, and **C:** 60 min) and frozen in quartz tubes by immersion in liquid nitrogen (77K). The EPR spectra were recorded at 77K.

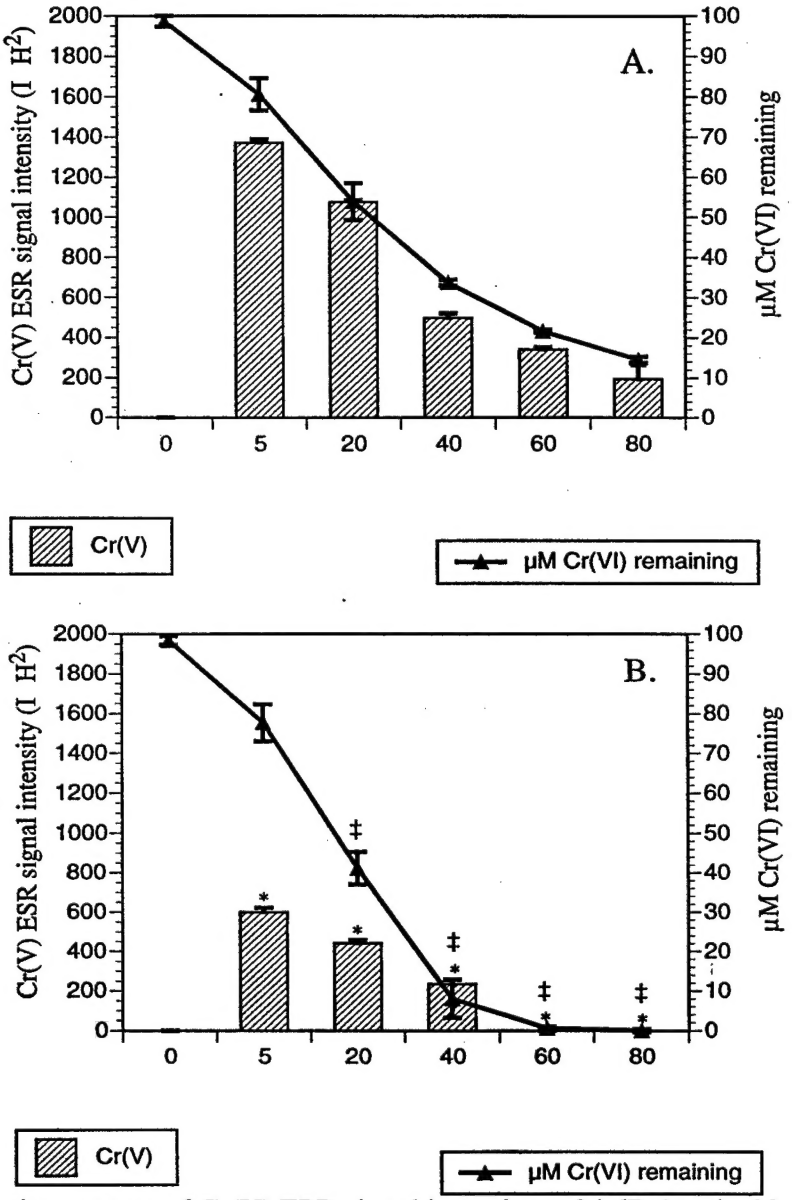


Fig. 7. Anaerobic time course of Cr(V) EPR signal intensity at 34 dB (vertical bars) relative to the level of Cr(VI) remaining (s), using proteoliposomes containing cytochrome b_5 and P450 reductase, 100 µM initial Cr(VI), and an NADPH-generating system. The EPR settings were as described in the Methods section. **(A):** Proteoliposomes plus the NADPH-generating system. **(B):** same as A, but includes the addition of 12.4 µM FeADP. (* Cr(V) levels that were statistically different in panel A vs B, $p < 0.015$) (‡ Cr(VI) levels that were statistically different in panel A vs B, $p<0.024$)

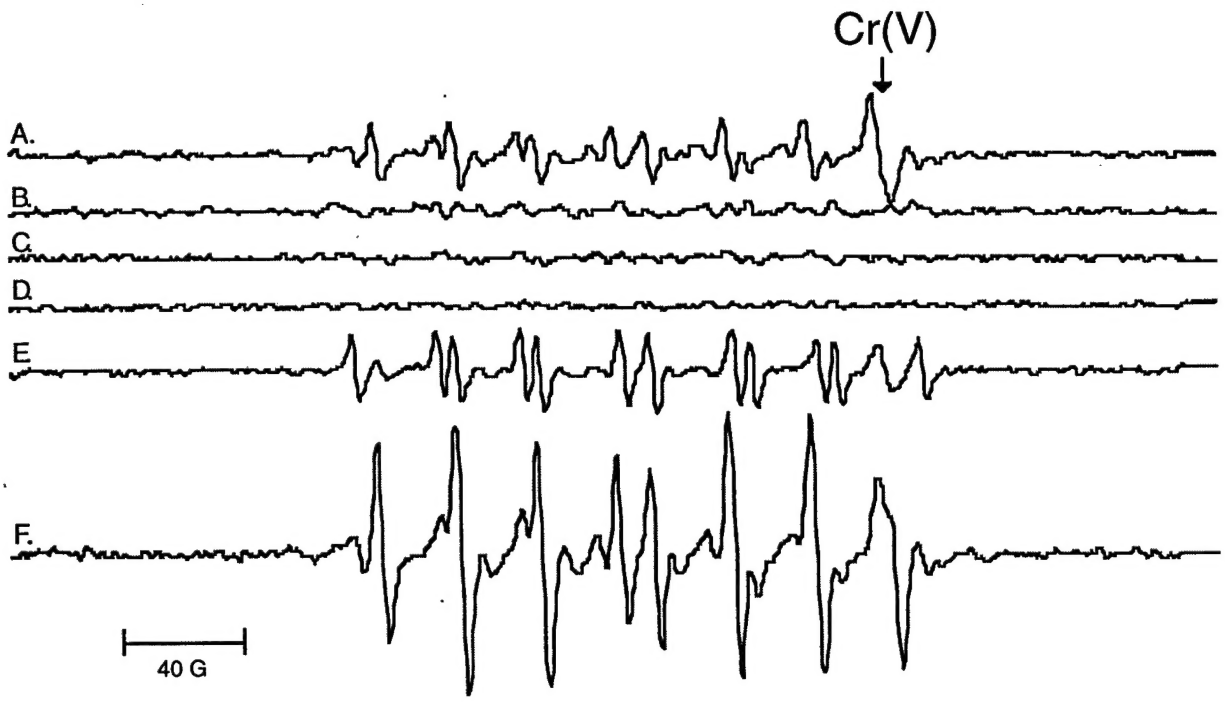
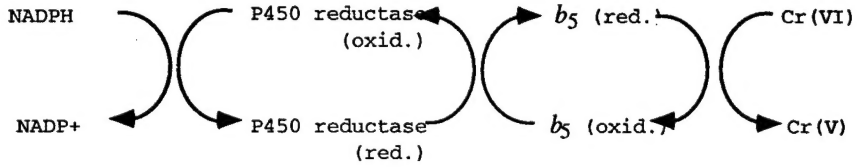


Fig. 8. ESR spectra obtained during the NADPH-dependent Cr(VI) reduction at 37°C by proteoliposomes under room air in the presence of DEPMPO (50 mM). **A:** Proteoliposomes containing b_5 + P450 reductase, 400 μM Cr(VI), and the NADPH-generating mix. **B:** Same as **A**, minus $NADP^+$. **C:** Same as **A**, minus Cr(VI). **D:** Same as **A**, except with catalase (5200 U). **E:** Same as **A**, except with ammonium formate (250 mM). **F**) Same as **A**, except with H_2O_2 (400 μM). Similar spectra were obtained when substituting human microsomes for proteoliposomes (not shown). For all, the total volume was 0.2 ml in K_2HPO_4 (pH 7.35)/ 3 mM $MgCl_2$/0.35 M KCl. The reactions were immediately transferred to a quartz mini-flat cell and placed in the ESR cavity. Spectra were recorded every 1.5 min; the spectrometer settings were: field set = 3210 G, sweep width = 200 G, time constant = 0.128 s, scan time = 1 min, modulation amplitude = 1 G, modulation frequency = 100 KHz, receiver gain = $2X10^4$, microwave frequency = 9.023 GHz, and microwave power = 20 mW.

A.



B.

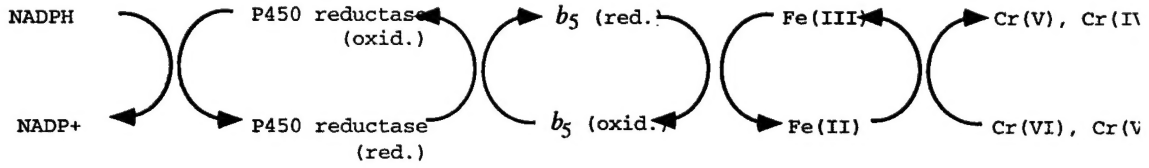


Fig. 9. The NADPH-dependent Cr(VI) reduction pathway in human microsomes, including potential redox cycles with Fe. **(A):** Cytochrome b_5 directly reduces Cr(VI). **(B):** Cytochrome b_5 directly reduces Fe(III) to Fe(II) which can reduce Cr(VI) and Cr(V).